Effects of Supplemental Chromium on Production of Cytokines by Mitogen-Stimulated Bovine Peripheral Blood Mononuclear Cells[1]

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ABSTRACT

This study determined whether supplementing the diets of dairy cows during the peripartum period with organic trivalent Cr influenced the capacity of their peripheral blood mononuclear cells to produce activation cytokines in response to stimulation with mitogens in vitro. Nine cows were fed 0.5 ppm of Cr/d per cow from 6 wk prepartum to 16 wk postpartum; 10 other periparturient cows served as unsupplemented controls. Mononuclear leukocytes, enriched from peripheral blood during wk 0, 2, 4, and 6 of lactation, were cultured with or without the T-lymphocyte mitogen, concanavalin A. Culture supernatants, harvested at 24, 48, or 72 h, were assayed for interleukin-2, interferon-γ, and tumor necrosis factor-α. The cytokines were barely detectable in the supernatants from the unstimulated cultures, but supernatants from mitogen-stimulated cultures contained higher concentrations of each cytokine. For cows fed Cr, concentrations of all three cytokines in the culture supernatants of the mitogen-stimulated mononuclear cells decreased significantly relative to values for unsupplemented cows, particularly around peak lactation for the 24- and 48-h cultures. Theses results extended our previous observations and supported the hypothesis that organic Cr is immunomodulatory in high producing cows.

(**Key words**: chromium, cytokines, mononuclear cells, immunomodulation)

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[1]Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

Abbreviation key: **Con A** = concanavalin A, **IFN** = interferon, **IL** = interleukin, **PBMC** = peripheral blood mononuclear cell, **PBST** = PBS containing Tween 80, **PBST+G** = PBST and gelatin, **rb** = recombinant bovine, **TNF-α** = tumor necrosis factor-α.

INTRODUCTION

Trivalent organic Cr is an essential micronutrient that is required for normal metabolism of carbohydrates, proteins, and lipids in humans and laboratory animals (2, 24). Certain stressors, such as strenuous exercise, carbohydrate loading, disease, and trauma, can induce Cr deficiency because each causes an increase in glucose metabolism that leads to mobilization of Cr from body reserves and irreversible loss of Cr in urine (3, 4, 5, 6). Chromium deficiency induces symptoms similar to those of diabetes in rats (26), but Cr supplementation dramatically increases their median and maximal lifespans (16). Chromium supplementation of human patients has improved glucose tolerance in type II diabetes, lowered blood low density lipoprotein cholesterol, and reduced body fat content (22). These beneficial actions of supplemental Cr have traditionally been attributed to a general improvement in insulin sensitivity and glucose tolerance in peripheral tissues (22, 25, 28). However, a more recent and intriguing hypothesis is that long-term Cr supplementation actually reverses the tendency for the brain to resist insulin as age increases, thus improving hypothalamic and pituitary function and associated neuroendocrine regulation of glucose homeostasis, including appetite control, intermediary metabolism, and thermoregulation (23). In either scenario, Cr probably acts by facilitating insulin receptor binding (24, 25), rate of insulin internalization (15), or both. Another intriguing possibility is that supplemental Cr increases longevity and retards

the aging process by improving immune function and enhancing resistance to infectious diseases.

Substantial evidence supports the theory that cows stressed by high milk production and husbandry might be susceptible to Cr deficiency. Feeder calves that had been stressed by transport but that had been supplemented with organic Cr upon entry to the feedlot had superior rates of gain and feed efficiency, decreased morbidity, and enhanced immune status compared with those for unsupplemented calves (9, 12, 13, 27, 29, 38, 39). Supplemental Cr also reduces the insulin intolerance that is typically observed in high producing, periparturient dairy cows (35) and enhances certain antibody and cellular immune response profiles relative to those of unsupplemented control cows (8). In particular, when peripheral blood mononuclear cells (**PBMC**) from feedlot calves that were stressed by transport (12) and PBMC from high producing periparturient dairy cows (8) were cocultured with concanavalin A (**Con A**), a T-lymphocyte mitogen, blastogenic responses by PBMC were significantly higher for groups supplemented with Cr than for the control groups. Furthermore, when blood sera from the periparturient dairy cows fed Cr were added to cultures of PBMC stimulated with Con A, suppression of blastogenesis associated with parturition was not only prevented, but blastogenic responses were actually enhanced relative to those for corresponding blood sera from control periparturient cows (11). The immunoenhancing activity of sera from cows supplemented with Cr could not be explained by typical endocrine factors related to glucose metabolism because serum concentrations of insulin, growth hormone, IGF-I, and tumor necrosis factor-α (**TNF-α**) were the same whether cows were supplemented with Cr or were left unsupplemented (11). However, those studies did not address the possibility that Cr affected blastogenic activity by altering cytokines produced by activated PBMC. Three cytokines produced during the early stages of PBMC activation are interleukin (**IL**)-2, interferon (**IFN**)-γ, and TNF-α.

The hypothesis of the present study was that Cr would influence the production of cytokines by lectin-activated PBMC. This hypothesis was tested by determining whether differences existed in concentrations of IL-2, IFN-γ, or TNF-α in supernatants of Con A-stimulated cultures of PBMC that were collected from cows supplemented with dietary Cr.

MATERIALS AND METHODS

Cows, Treatments, and Blood Collection Schedules

As described previously (8, 19, 20), periparturient Holstein cows were included in this study. Cows were housed at the University of Guelph, Elora Dairy Research Centre (Elora, ON, Canada) and were randomly allocated across two Cr supplementation groups (9). Briefly, 9 cows received diets supplemented with 0.5 ppm/d per cow of chelated Cr (Metalosate, 2.68% Cr; Sureleen-Albion Agra, Inc., Arva, ON, Canada) from wk 6 prior to the expected calving date to wk 16 of lactation, and 10 cows received unsupplemented diets during this treatment period. Prior to calving, cows were fed a total mixed diet for ad libitum intake, and the Cr was supplemented daily in a top-dressing containing 205 mg of Metalosate (approximately 5.49 mg of Cr) in 200 g of ground corn. From parturition through wk 6 of lactation, cows were fed a total mixed diet twice daily, and a top-dressing of 185 mg of Metalosate in 100 g of ground corn was provided to cows receiving the Cr supplement at each feeding. Dietary formulations for dry and lactating cows also have been described previously (8). Cows were milked twice daily at 0500 and 1500 h. Sixty milliliters of peripheral blood were collected by venipuncture (middle coccygeal vein) into sterile, heparinized vacutainer tubes (Fisher Scientific, Don Mills, ON, Canada) on wk 0, 2, 4, and 6 of lactation. This blood was used as the source of PBMC for culture with Con A and subsequent collection of culture supernatants for cytokine assays.

PBMC Cultures

The PBMC were enriched from heparinized blood samples by density gradient centrifugation (Histopaque 1.077; Sigma Chemical Co., St. Louis, MO) as described previously (10). The PBMC were resuspended to a final concentration of 2×10^6 cells/ml in supplemented RPMI 1640 culture medium [10% heat-inactivated fetal bovine serum (Flow Laboratories, Mississauga, ON, Canada), 200 m*M* L-glutamine (Sigma Chemical Co.), and 100 IU of penicillin and streptomycin (Central Media Laboratory, Ontario Veterinary College, University of Guelph, Guelph, ON, Canada)]. In parallel cultures with those reported in a previous study (8), 40 μl of cell suspension from each sample were inoculated into 24 wells on three separate 96-well, flat-bottomed microtiter culture plates (Flow Laboratories). The first plate was used for a 24-h incubation, the second for a 48-h incubation, and the third for a 72-h incubation. Sixty microliters of culture medium were added to the first 12 wells of each plate (unstimulated cells), and 60 μl of culture medium containing 10 μg/ml of Con A (Sigma Chemical Co.) were added to the second set of 12 wells. Plates were incubated at 37°C in 5% CO_2 and moist air. After 24, 48, or 72 h, plates were centrifuged (600 $\times$ *g* for 10 min at 25°C) to pellet cells, and 100 μl of supernatant from each of the 12

wells per sample group (1200 μl per sample) were collected into 1.5-ml sterile microcentrifuge tubes. The tubes were shipped to the National Animal Disease Center (Ames, IA) and stored at –60°C until assayed for cytokines.

Cytokine Assays

IL-2 bioassay. The IL-2 concentrations in culture supernatants were measured by a bioassay using the bovine 300B1 T cell clone (34) (kindly provided by Mark Stevens, USDA-ARS, National Animal Disease Center, Ames, IA). The 300B1 cells, when stimulated with γ-irradiated bovine PBMC as antigen-presenting cells, proliferated in response to IL-2 or IL-1 when costimulated with Con A (34). In the present study, concentrations of IL-2 were assayed in 24- and 48-h supernatants because IL-2 synthesis in response to Con A declines rapidly after 24 h (37). The γ-irradiated PBMC and 300B1 cells were separated by centrifugation (400 × g for 30 min at 25°C) over Histopaque 1083 (Sigma Chemical Co.). Recovered 300B1 cells were washed (650 × g for 10 min at 25°C) three times in PBS. The 300B1 cells were resuspended to 4×10^5 viable cells/ml in RPMI 1640 medium supplemented with 25 m*M* HEPES buffer (Sigma Chemical Co.), 2 m*M* L-glutamine (Sigma Chemical Co.), 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), 100 μ*M* minimum essential medium nonessential amino acids (Sigma Chemical Co.), 50 μ*M* 2-mercaptoethanol (Sigma Chemical Co.), and 1% antibiotics (100 U of penicillin/ml, 100 μg of streptomycin/ml, and 0.25 μg of amphotericin B/ml; Sigma Chemical Co.) (complete medium). One hundred microliters of recombinant human IL-2 standard (0, 0.1, 0.5, 1, 1.5, and 2 U/ml; Genzyme Diagnostics, Cambridge, MA) or 24- or 48-h test culture supernatants were added to triplicate wells of 96-well, flat bottom microtiter plates. The Con A activity of test culture supernatants was inhibited by adding methyl α-D-mannopyranoside (10 mg/ml, final concentration per well), and IL-1 activity was blocked (33) by adding a human IL-1 receptor antagonist (R&D Systems, Minneapolis, MN) to each well (50 ng/ml, final concentration per well). Finally, 50 μl of 300B1 cell suspension (2×10^4 cells per well) were added to each well of each plate, and cultures were incubated for 48 h at 37°C in 5% CO_2 and moist air. Cultures were then pulsed for 18 h with 1 μCi of [methyl-^{3}H]thymidine (Amersham Life Science, Arlington, Heights, IL) per well in 50 μl of complete medium and harvested onto glass fiber filters (PHD Cell Harvester; Cambridge Technology, Watertown, MA). Retained radioactivity was counted by liquid scintillation spectrophotometry (model hS800D; Beckman Instruments Inc., Fullerton, CA) and recorded as counts per minute. The concentrations (units per milliliter) of IL-2 in 24- and 48-h test culture supernatants were calculated from two separate standard curves (Figure 1a and 1b) that were created by regressing counts per minute on the concentrations of the IL-2 standards.

IFN-γ ELISA. Interferon-γ was measured in supernatants at 24, 48, and 72 h. Because IFN-γ in culture supernatants from unstimulated cells was undetectable in previous studies and in random samples from the current study, only a small random sample of unstimulated supernatants was assayed, which served as a negative control in assays of Con A-stimulated supernatants.

Interferon-γ was measured as previously described (1, 36) using an IFN-γ capture ELISA (protocol and reagents generously provided by Dale Godson, Veterinary Infectious Disease Organization, Saskatoon, SK, Canada). Assays were performed in Immunolon II microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA). Reagents consisted of a capture antibody [mouse anti-recombinant bovine (**rb**) IFN-γ, IgG fraction], detection antibody (rabbit anti-bovine IFN-γ, IgG fraction), rbIFN-γ, biotinylated goat anti-rabbit IgG (Zymed Laboratories, Inc., South San Francisco, CA), horseradish peroxidase-conjugated streptavidin-biotinylated complex (Amersham Corp., Arlington Heights, IL), and substrate [N-ethylbenzothiazolinone-6-sulfonic acid azine in citrate buffer and H_2O_2 at 0.1%, vol/vol]. Capture monoclonal antibodies were screened initially according to their reactivity to rbIFN-γ coated on ELISA plates; positive clones were then tested for their ability to inhibit IFN-γ activity induced by antiviral and major histocompatibility class II antigens. Clone 2-2-1 was selected for further testing because it was able to inhibit activities induced by both antigens. Ascites fluid from clone 2-2-1 also had a no blocking effect for IFN-α and reacted in Western blots with rbIFN-γ but not with rbIFN-α, TNF-α, or IL-2. Specificity of the polyclonal detection antibody was similarly confirmed by its ability to block INF-γ effects caused by antiviral and major histocompatibility class II antigens and its lack of effect on interferon-α activity (Dale Godson, 1996, personal communication).

Internal standards consisting of serially diluted rbIFN-γ were prepared in PBS containing Tween 80 (**PBST**; 0.1%, vol/vol) and PBST plus gelatin (**PBST+G**; 0.1%, vol/vol). Positive and negative control samples and test samples were also serially diluted in PBST+G. Capture antibody was diluted

1:4000 (vol/vol) in carbonate-coating buffer, and detection antibody was diluted 1:1000 (vol/vol) in PBST+G. Biotinylated goat anti-rabbit IgG and horseradish peroxidase-conjugated streptavidin-biotinylated complex were diluted in PBST [1:10,000 (vol/vol) and 1:2000 (vol/vol), respectively]. Intervening washes were conducted using PBST. Absorbance of standards, controls, and test samples were read at 405 and 490 nm using an automated ELISA plate washer and reader (Dynatech MR7000; Dynatech Laboratories Inc., Guernsey Channel Islands, UK). Interferon-γ in test samples was determined from a standard curve (Figure 1c) of absorbance regressed on IFN-γ concentration for the dilution of the test sample that produced absorbance readings in the linear portion of the curve. The IFN-γ concentration in culture supernatants was calculated by multiplying the value from the standard curve by the dilution factor and was expressed in nanograms per milliliter.

TNF-α radioimmunoassay. The TNF-α radioimmunoassay was the last to be performed in this

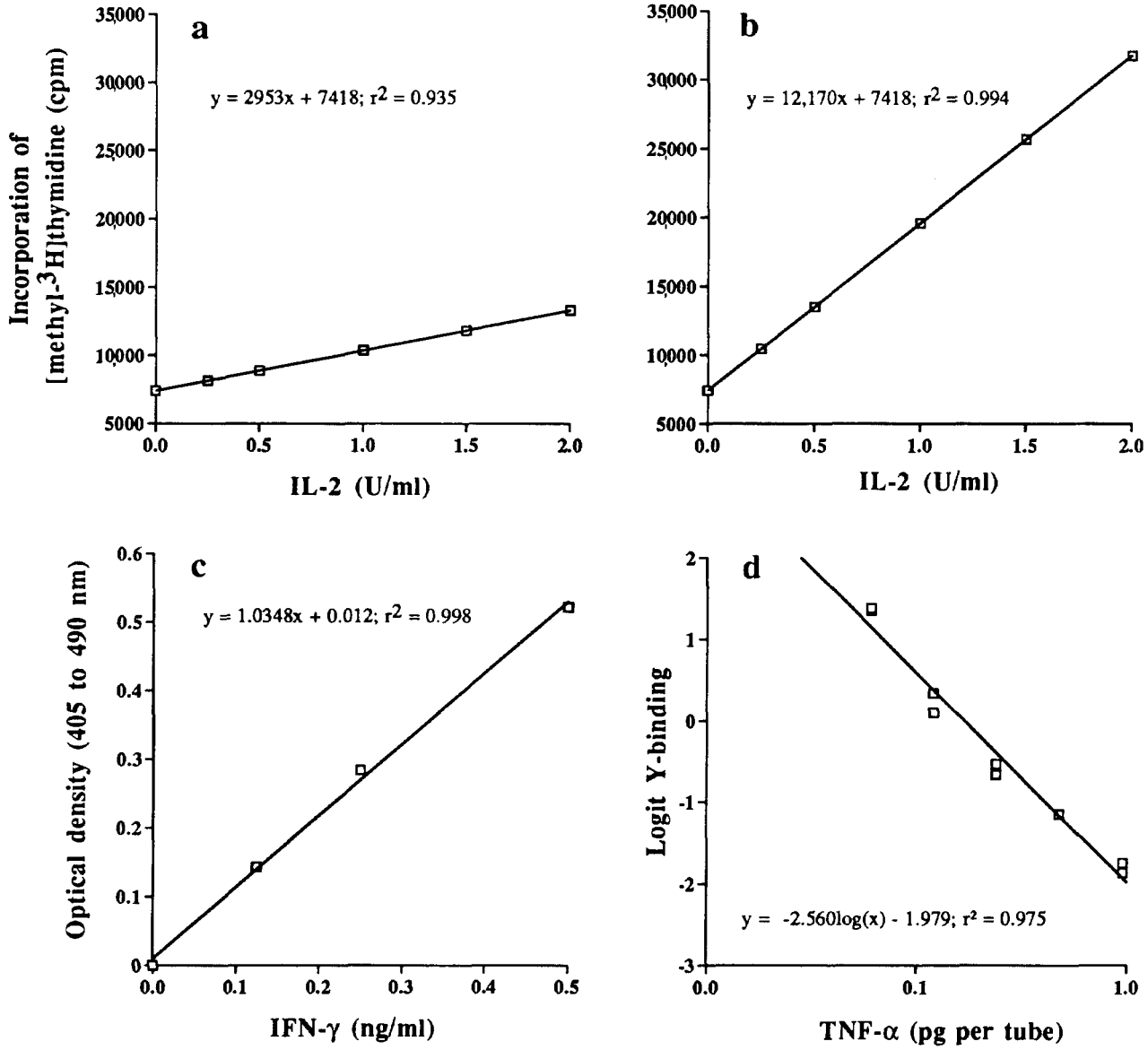

Figure 1. Standard curves used to obtain data from the interleukin-2 (IL-2) bioassay (a, 24-h culture supernatants; b, 48-h culture supernatants), the interferon-γ (IFN-γ) ELISA (c), and the tumor necrosis factor-α (TNF-α) radioimmunoassay (d). The r^2 values for the linear curves were all >0.92, and all test data fell within the linear part of each curve.

study. Because this assay had to be run in duplicate using approximately 500 μl of culture supernatant per sample, we did not have a great enough volume of supernatants left over from the IL-2 and IFN-γ assays to analyze TNF-α in individual samples. Therefore, 100 μl of supernatant from individual cows were pooled by treatment group (Cr or control) for each week of lactation (wk 0, 2, 4, and 6) and for each culture period (24, 48, and 72 h). As a consequence, a total of 24 supernatants from Con A-stimulated cultures and 24 supernatants from unstimulated cultures were analyzed in duplicate. Immunoreactive TNF-α in the supernatants was assayed as described by Kenison et al. (21) in one assay performed on a single day. Equivalent volumes of RPMI-1640 medium were added to the standards and to nonspecific binding tubes to compensate for matrix effects. The concentration of TNF-α in test supernatants was calculated from a standard curve (Figure 1d) created by regressing radioactivity (counts per minute) on TNF-α concentrations of the standards. Assay sensitivity was 48 pg/ml, and the intraassay coefficient of variation was 2.3%.

Statistical Methods

Data were assessed for normality of distributions prior to statistical analysis using the univariate procedure of SAS. Results of this analysis showed near normal distributions for all three cytokine data files, which were not significantly improved with $\log_{10}$ transformations. Therefore, only untransformed data were analyzed by least squares means using the general linear models procedure of SAS.

The statistical models always included treatment group (supplemented vs. unsupplemented), week relative to parturition, and an interaction term of week and treatment. For the IL-2 and IFN-γ data files, which included observations from individual cows, cow(treatment) was included as a random effect, and the ANOVA used the expected mean square for this source of error during F tests of the treatment effect. All other effects were tested against the residual error mean square. The residual error mean square was also used as the error term during hypothesis tests of the TNF-α data, because pooling of culture supernatants by treatment group for each lactational and PBMC culture sample time eliminated the cow effect. Data presented in Figures 2 and 3 are the least squares means of the interaction of week and treatment resulting from the ANOVA of IL-2 and IFN-γ data. The TNF-α data (Figure 4) are presented as means of lactation week over all incubation times or as means of incubation time over all lactation weeks. Indicated significant differences between treatment means were taken from the matrix of paired t tests associated with each set of least squares means. Significance was determined at $P \leq 0.05$.

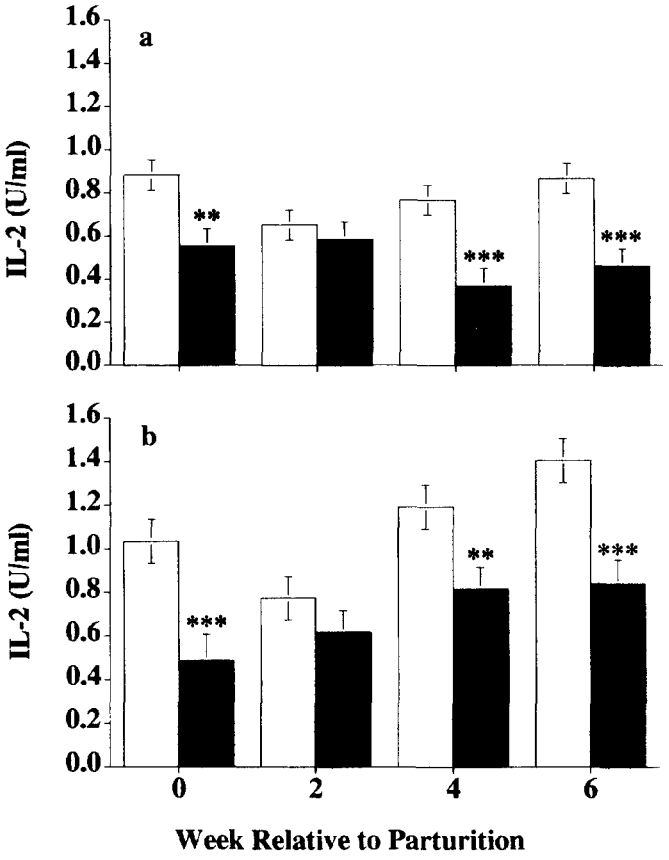

Figure 2. Concentrations of interleukin-2 (IL-2) in 24-h (a) and 48-h (b) culture supernatants of concanavalin A-stimulated peripheral blood mononuclear leukocytes from periparturient cows supplemented with Cr (■) or unsupplemented (control; □). Significant differences from controls (**$P < 0.05$; ***$P < 0.01$).

RESULTS

Effects of Supplemental Cr on IL-2

Interleukin-2 in 24- and 48-h culture supernatants from unstimulated PBMC was barely detectable (weekly means ranged from 0.05 to 0.20 U/ml ± 0.04 SEM), and neither treatment nor the interaction of week and treatment contributed to the variation in these data (data not shown). This result was in contrast to the result for IL-2 concentrations in culture supernatants from Con A-stimulated PBMC that approached 0.66 U/ml after 24 h of culture and aver-

aged roughly 0.93 U/ml after 48 h (Table 1). Most of the variation in these IL-2 data was accounted for by the cow(treatment) effect, but treatment also contributed to the variation (Table 1). Chromium supplementation was associated with overall reductions in 24-h (0.49 ± 0.04 vs. 0.79 ± 0.03; $P = 0.02$) and 48-h (0.69 ± 0.06 vs. 1.10 ± 0.05; $P = 0.05$) IL-2 concentrations relative to control values. Least squares means of the interaction of week and treatment showed that this effect occurred first at parturition (wk 0) and again around peak milk production (wk 4 and 6) when IL-2 concentrations from the control group were highest (Figure 2).

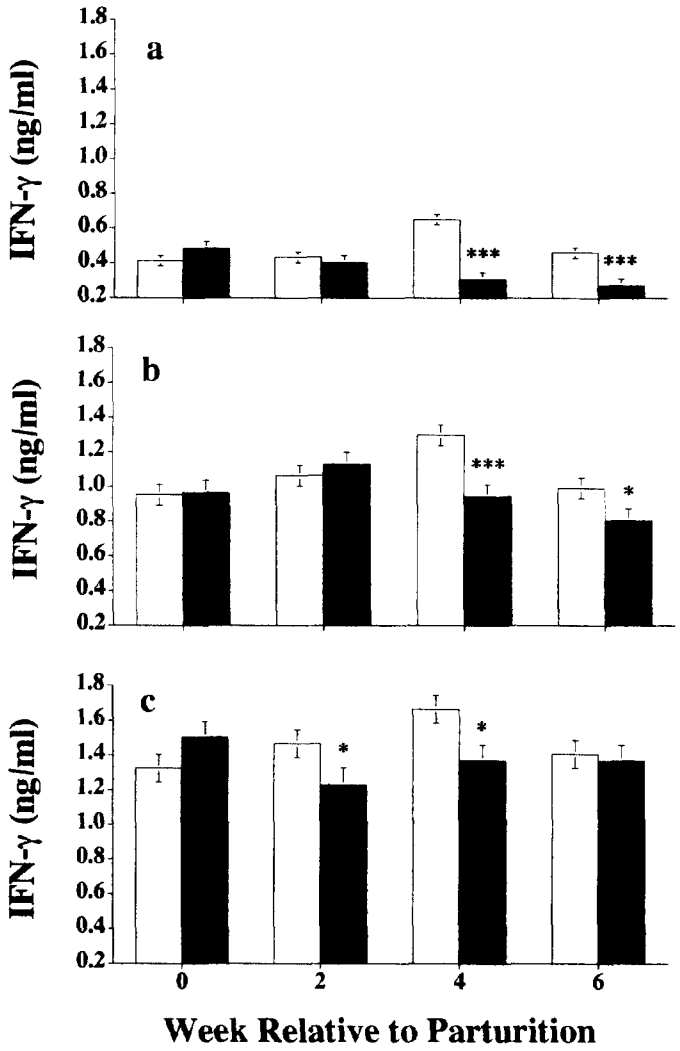

Figure 3. Concentrations of interferon-γ (IFN-γ) in 24-h (a), 48-h (b), and 72-h (c) culture supernatants of concanavalin A-stimulated peripheral blood mononuclear leukocytes from periparturient cows supplemented with Cr (■) or unsupplemented (control; □). Significant differences from controls (*$P < 0.10$; ***$P < 0.01$).

Effects of Supplemental Cr on IFN-γ

Interferon-γ in unstimulated culture supernatants was undetectable. However, IFN-γ concentrations in supernatants from Con A-stimulated cultures increased with incubation time and reached peak

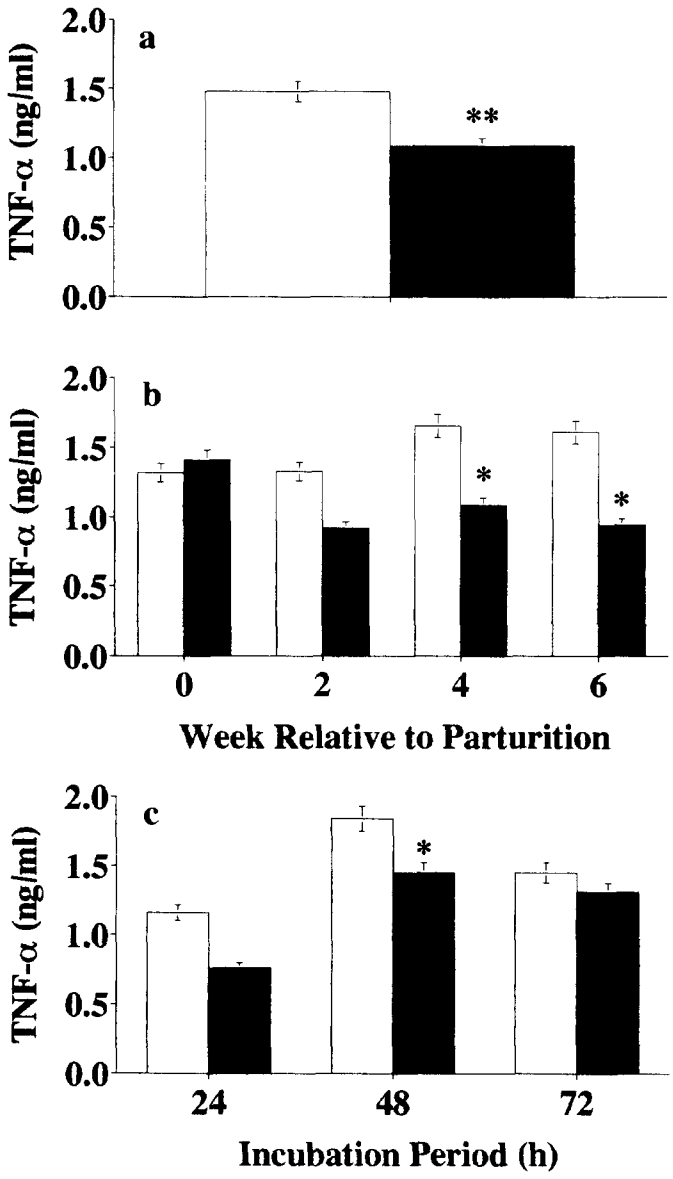

Figure 4. Concentrations of tumor necrosis factor-α (TNF-α) in pooled (by treatment group for each sample week) culture supernatants of concanavalin A-stimulated peripheral blood mononuclear leukocytes from periparturient cows supplemented with Cr (■) or unsupplemented (control; □). Data presented are the overall treatment means (a) the means over all incubation times for the interaction of week and treatment (b), and the hourly means over all sample weeks for the interaction of week and treatment (c). Significant differences from controls (*$P < 0.10$; **$P < 0.05$).

TABLE 1. Results of ANOVA of cytokine concentrations in culture supernatants of concanavalin A-stimulated peripheral blood mononuclear cells from periparturient dairy cows that were fed diets supplemented with Cr or not supplemented.[1]

Model parameter	IL-2		IFN-γ			TNF-α
	24 h	48 h	24 h	48 h	72 h	
Model R^2	0.46	0.48	0.66	0.67	0.63	0.75
Mean	0.6588	0.9340	0.4378	1.0289	1.4279	1.2848
Model CV	56.47	57.82	32.64	26.52	23.78	23.63
Effects in model	Type III SS (*P*)					
Treatment	0.0237	0.0547	0.1259	0.4598	0.6182	0.0082
Cow(treatment)[2]	0.0001	0.0001	0.0001	0.0001	0.0001	. . .
Sample week	0.2102	0.0001	0.0142	0.0025	0.2338	0.5009
Week × treatment	0.0739	0.1789	0.0001	0.0082	0.0247	0.1898
Incubation hour[3]	. . .	. . .	. . .	. . .	. . .	0.0082
Treatment × hour[3]	. . .	. . .	. . .	. . .	. . .	0.2986

[1]IL-2 = Interleukin-2, IFN-γ = interferon-γ, and TNF-α = tumor necrosis factor-α (measured on samples pooled over individual cows by treatment group, lactation week, and incubation time of peripheral blood mononuclear cells).

[2]Cow(treatment) was a random effect in the statistical model used in ANOVA of the IL-2 and IFN-γ data files but was not included in the statistical model for the TNF-α data analysis because culture supernatants from individual cows were pooled by treatment group for each lactation week and each incubation time of peripheral blood mononuclear cells.

[3]Effects of incubation hour and the interaction of treatment and incubation hour were only tested for the pooled TNF-α data file.

values at 72 h. Twenty-four-, 48-, and 72-h means were 0.43, 1.02, and 1.43 ng/ml ± 0.03 SEM, respectively. Regardless of culture time, cow (treatment) and the interaction of week and treatment explained most of the variation in IFN-γ data files. The overall treatment effect was not significant for any culture time (Table 1). Plots of least squares means of the interaction term showed that Cr reduced Con A-induced IFN-γ production by PBMC when cells were collected from cows around peak milk production (wk 4 and 6) and when cells were coincubated with mitogen for 24 h (Figure 3a) and 48 h (Figure 3b). By 72 h in culture, IFN-γ production by PBMC from both treatment groups had increased, and only modest reductions in concentration were detected during wk 2 and 4 for the group supplemented with Cr (Figure 3c).

Effects of Supplemental Cr on TNF-α

Concentrations of TNF-α in pooled supernatants from unstimulated PBMC were very low (0.15, 0.18, and 0.18 ng/ml ± 0.01 SEM for 24-, 48-, and 72-h cultures, respectively) and did not vary with treatment, sample week, or hour in culture (data not shown). This result was in contrast to mean TNF-α concentrations following Con A stimulation of the PBMC, which were well above 1.0 ng/ml (Table 1). Although pooling individual cow supernatants by treatment group precluded testing a cow effect, ANOVA of these data determined that both treatment and hours of incubation contributed significantly to the variation in TNF-α concentrations induced by Con A (Table 1). A plot of the overall treatment means (Figure 4a) showed that the TNF-α concentration was lower ($P = 0.008$) when PBMC were collected from cows fed supplemental Cr than when PBMC were collected from unsupplemented cows, and means of the interaction of week and treatment (Figure 4b) determined that this effect was most significant around peak milk production (wk 4 and 6). Although trends for reduced TNF-α secretion associated with Cr supplementation were present for all incubation times, statistical significance was observed only for the 48-h cultures (Figure 4c).

DISCUSSION

Based on previously published observations indicating that the blastogenic responses of Con A-stimulated PBMC were higher in cells collected from cattle supplemented with Cr than from unsupplemented cattle (8, 12), the present study examined whether supplementation of cows with Cr affected cytokine production by mitogen-stimulated PBMC. The three cytokines assayed for in the present study (IL-2, IFN-γ, and TNF-α) are intimately involved in early up-regulation of cellular immune responses (14), and IL-2 and IFN-γ are produced by mitogen-stimulated PBMC from cattle (B. J. Nonnecke, 1994, unpublished data).

Wienberg et al. (37) showed that Con A-induced IL-2 production by bovine lymph node cells in vitro was biphasic; peak IL-2 synthesis occurred between 0 to 4 h and again at 8 to 18 h. Secretion declined

rapidly after 18 h, and secretion was negligible after 24 h. The IL-2 produced during the first 4 h of the culture period accounted for up to 40% of the total IL-2 produced during the first 18 h. Concanavalin A and IL-2 also induced IL-2 receptor expression on T cells before increases in blastogenic rate were detectable (32).

The IL-2 concentrations in the present study were significantly lower in cultures of PBMC from cows fed Cr than in cultures of PBMC from unsupplemented cows (Figure 2). Blastogenic responses of identical cell cultures from the cows supplemented with Cr were previously shown to be higher than those of cells cultured from the unsupplemented cows (8). One possible explanation of our combined results is that Cr supplementation caused early (before 24 h) elevated sensitivity of PBMC to Con A and, therefore, more rapid activation, resulting in accelerated production and subsequent utilization of IL-2. If this theory were true, IL-2 concentrations in 24- and 48-h cultures of PBMC from cows supplemented with Cr would be less than concentrations in the corresponding cultures using cells from unsupplemented cows. It was not possible to assay IL-2 or to monitor IL-2 receptor expression during the initial 24 h of the incubation period in the present study; however, assessment of protein secretion and receptor expression during the early stages of PBMC activation could help clarify mechanisms by which Cr influences PBMC physiology. Chromium causes general improvement in insulin sensitivity and glucose tolerance in peripheral tissues (22, 25, 28), increases fluidity of membranes of synthetic liposomes (15), and enhances the rate of internalization of insulin and the uptake of glucose and leucine in rat muscle cells (15). These observations suggest that insulin-mediated mechanisms of PBMC activation, including IL-2 production and utilization, should be considered in future studies regarding the immunomodulatory role of Cr.

A final observation from IL-2 data was that IL-2 concentrations of the present study were higher in 48-h supernatants than in 24-h supernatants (Figure 2). Our results, although disconcerting given the results of Weinberg et al. (37) that IL-2 secretion is negligible after 24 h, can be explained. The 24- and 48-h supernatants were assayed on separate days, necessitating the use of two separate standard curves. These standard curves had different slopes and Y-intercepts, resulting in lower IL-2 concentrations in 24-h supernatants than in 48-h supernatants (Figures 1a and 1b). Although the kinetics of IL-2 secretion could not be assessed accurately in the present study, the original null hypothesis was able to be tested. Data in Figure 2 clearly demonstrate that supplemental dietary Cr decreased the concentration of IL-2 in 24- and 48-h supernatants from cultures of Con A-stimulated cells. These data also demonstrate that the effect of Cr on IL-2 secretion was most pronounced around parturition (wk 0) and during peak milk production (wk 4 and 6). Interestingly, blastogenic responses of the cells were also most affected during these periods (8).

Interferon-γ and TNF-α mRNA reach peak accumulation in human PBMC between 12 and 24 h following mitogenic stimulation (19) and, therefore, act later than does IL-2. Interleukin-2 also regulates the synthesis of IFN-γ (32) and TNF-α (31) by activated T-lymphocytes. In cattle, depression of PBMC IFN-γ production that is induced by parturition can be augmented by addition of IL-2 to the culture medium (20). Therefore, because IL-2 concentrations were reduced in 24- and 48-h supernatants of PBMC cultures from cows supplemented with Cr in the present study, it was not surprising that IFN-γ (Figure 3) and TNF-α concentration (Figure 4) were also reduced. Although the concentrations of IFN-γ was highest in 72-h cultures (Figure 3c), Cr effects were most obvious in 24- and 48-h cultures (Figure 3, a and b, respectively), particularly during peak milk production (wk 4 and 6). Effects of Cr on TNF-α were also most obvious at peak production (Figure 4b) and were significant overall in 48-h cultures (Figure 4c). Lowered secretion of IFN-γ and TNF-α by PBMC was consistent with the elevated blastogenesis reported in previous studies (8, 11, 12) because both of these cytokines have known antiproliferative activities in mitogen-stimulated hematopoietic (7, 18) and lymphoid cells (17). Furthermore, lowered concentrations of TNF-α in cultures of cells from cows fed the Cr supplement were consistent with observations by Wright et al. (38), who showed that feedlot calves receiving Cr supplement had lower blood haptoglobin concentrations than did unsupplemented calves. Haptoglobin is an acute phase protein produced by the liver in response to elevated blood TNF-α concentrations that suppresses bovine PBMC function (30).

CONCLUSIONS

It is not known whether Cr directly affects the immune system or acts through indirect pathways that alter tissue sensitivities to glucose-regulating hormones such as cortisol, insulin, growth hormone, or IGF-I or to acute phase cytokines such as IL-2, IFN-γ, and TNF-α. Future studies to evaluate the

effects of Cr on the function of the bovine immune system would benefit from protocols that include examination of possible direct effects of Cr on leukocytes and effects of Cr supplementation on responsiveness to vaccination and resistance to pathogens of clinical relevance. In this regard, preliminary results (29) are encouraging; they suggest that organic Cr can dramatically reduce morbidity of cattle that have undergone stressful husbandry procedures.

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